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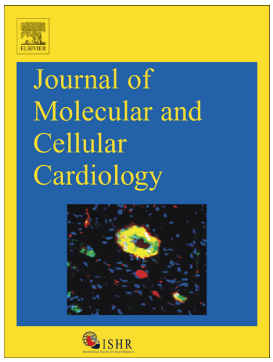

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Cartilage oligomeric matrix protein is a novel notch ligand driving embryonic stem cell differentiation towards the smooth muscle lineage

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Running title: *COMP directs ESC-VSMC differentiation*

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Abstract

Cartilage oligomeric matrix protein (COMP), a protective component of vascular extracellular matrix (ECM), maintains the homeostasis of mature vascular smooth muscle cells (VSMCs). However, whether COMP modulates the differentiation of stem cells towards the smooth muscle lineage is still elusive. Firstly, purified mouse COMP directly induced mouse embryonic stem cell (ESC) differentiation into VSMCs both *in vitro* and *in vivo*, while the silencing of endogenous COMP markedly inhibited ESC-VSMC differentiation. RNA-Sequencing revealed that Notch signaling was significantly activated by COMP during ESC-VSMC differentiation, whereas the inhibition of Notch signaling attenuated COMP-directed ESC-VSMC differentiation. Furthermore, COMP deficiency inhibited Notch activation and VSMC differentiation in mice. Through silencing distinct Notch receptors, we identified that Notch1 mainly mediated COMP-initiated ESC-VSMC differentiation. Mechanistically, COMP N-terminus directly interacted with the EGF11-12 domain of Notch1 and activated Notch1 signaling, as evidenced by co-immunoprecipitation and mammalian two-hybrid assay. In conclusion, COMP served as a potential ligand of Notch1, thereby driving ESC-VSMC differentiation.

Keywords: Embryonic stem cells; Vascular smooth muscle cells; COMP; Notch signaling

1. Introduction

Embryonic stem cells (ESCs) with the feature of pluripotency have the potential to differentiate into vascular smooth muscle cells (VSMCs), which express specific cell markers, such as α-smooth muscle actin (α-SMA), smooth muscle protein 22α (SM22α), calponin and smooth muscle myosin heavy chain (SM-MHC) and fine-tune vascular integrity and homeostasis [1, 2]. Thus, ESCs serve as a promising source of VSMCs in vascular tissue engineering, angiogenesis, and vasculogenesis and play a pivotal role in vascular injury repair [3]. The downstream signals of cell surface receptors including integrins, Notch and growth factor receptors (e.g., TGFR, PDGFR) [4, 5], as well as Nrf3-mediated transcriptional regulation [6], Nox4-derived ROS production [1] and HDAC- or miRNA-related epigenetic modulation [7], have been identified to mediate this differentiation process, although the mechanism of stem cell differentiation into the smooth muscle lineage is not fully understood.

The extracellular microenvironment, composed of a variety of extracellular matrix (ECM) components, ECM-bound growth factors, receptors and proteases, is a crucial regulator of the cell fate of ESCs [8-11]. In particular, ECM proteins, including collagens, elastins, proteoglycans and glycoproteins, fine-tune cell differentiation and behavior via protein-protein complex interaction. Moreover, the constitution and stiffness of the ECM coordinately direct cell division and differentiation. Through recent proteomic analysis, more than 100 ECM proteins have been identified in human aortas and porcine stented coronary arteries, most of which are glycoproteins and proteoglycans with unknown functions [12, 13]. Nevertheless, only a few ECM proteins have been suggested to be involved in smooth muscle lineage specification. For example, vascular wall resident collagen IV and hyaluronan have been reported to direct ESC differentiation into SMCs [14, 15]. Exploring how ECM components mediate ESC differentiation towards the smooth muscle lineage would facilitate the development of new regenerative therapies for repairing injured VSMCs in vascular diseases.

Cartilage oligomeric matrix protein (COMP), a 524 kDa pentameric noncollagenous glycoprotein, is a matricellular protein that is abundant in the cardiovascular system. Our recent studies have shown that COMP plays protective roles in maintaining mature VSMC homeostasis by interacting with various binding proteins (e.g., integrin α7 and BMP-2) [16, 17]. COMP maintains the contractile phenotype of VSMCs and suppresses atherosclerotic neointima formation and VSMC calcification [16-18]. Here, we unexpectedly discovered that COMP may serve as a novel ligand of Notch1, thereby directly driving ESC differentiation towards the smooth muscle lineage.

2. Materials and methods

2.1 Materials

Recombinant platelet-derived growth factor-BB (PDGF-BB) (220-BB) was obtained from R&D (Flanders, NJ, USA), and transforming growth factor-β (TGF-β) (AF-100) was obtained from PeproTech Group Inc. (Chicago, IL, USA). Antibodies against COMP (ab28400), Notch1 (ab65297), SM α-actin (α-SMA) (ab119952), calponin (ab46794), and SM22α (ab10135) are from Abcam plc (Cambridge, UK). DAPT (orb65891) was purchased from Biorbyt (Cambridge, UK). Antibody against cleaved Notch1 (Val1744) was obtained from Cell Signaling Technology (Boston, MA, USA). Gelatin was obtained from Merck Millipore (Darmstadt, Germany). Heparin-agarose beads (H0402) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Matrigel™ Basement Membrane Matrix (356234) was bought from BD Biosciences (San Diego, CA, USA).

2.2 Cell Culture and ESC-VSMC Differentiation

Mouse ESCs (ES-D3 cell line, CRL-1934) were obtained from ATCC Inc. (Manassas, VA, USA). ESCs were seeded at density of 2×10^5 cells per 25 cm^2 on mitomycin C (10 μg/ml)-treated confluent mouse embryonic fibroblast (MEF) cell feeder in 0.1% gelatin (Millipore, Boston, MA, USA)-coated flasks and cultured in ESC medium including Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Carlsbad, FL, USA), 10% EmbryoMax fetal bovine serum (FBS) (Millipore, Boston, MA, USA), 10 ng/ml of leukemia inhibitory factor (LIF) (Millipore, Boston, MA, USA), 0.1 mM of 2-mercaptoethanol (2-ME) (Life Technologies, Carlsbad, FL, USA), 100 U/ml of penicillin (Life Technologies, Carlsbad, FL, USA), 100 μg/ml of streptomycin (Life technologies, Carlsbad, FL, USA) and 2 mM of glutamine (Life Technologies, Carlsbad, FL, USA). They were split at a ratio of 1:6 every other day and seeded at density of 2×10^5 cells per 25 cm^2. For ESC-VSMC differentiation, cell culture dishes were pre-coated with 0.5 ml of Tris-buffered saline containing various concentrations of gelatin, mouse collagen IV or mouse COMP per cm^2 area at room temperature overnight. Undifferentiated ESCs (2×10^5 cells per 60 mm dish) were seeded on mouse collagen IV (10 μg/ml)- or purified-COMP-coated dishes in differentiation medium (DM) containing α-minimal essential medium (Life Technologies, Carlsbad, FL, USA) supplemented with 10% FBS, 0.05 mM of 2-ME, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 2 mM of glutamine. DM was refreshed every day after the second day of differentiation. The cells were cultured in DM for 3-7 days, after which they were harvested and further analyzed. 293A cells and COS-7 cells from ATCC were maintained and passed in high-glucose DMEM supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin in an incubator with a 5% CO_2/water-saturated atmosphere at 37°C.

2.3 Recombinant COMP Expression in 293A Cells and COMP Purification

The plasmid pcDNA3.1 encoding cDNA of full-length mouse COMP was constructed and transfected into 293A cells using of Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA). Stably transfected cells were selected with 750 mg/L G418. For COMP purification, stably transfected 293A cells were incubated in serum-free Opti-MEM (GIBCO, Rockford, CA, USA) with G418 (250 mg/L) for 48 hours. The conditioned medium was incubated with heparin-agarose beads (Sigma-Aldrich, St. Louis, MO, USA)

equilibrated with Tris-buffered saline containing 2 mM of $CaCl_2$ for binding overnight at 4°C with gentle agitation. After five-time washes, COMP was eluted with 0.75 M NaCl (buffered in 10 mM Tris, pH 7.5) containing 2 mM of $CaCl_2$. The purified mouse COMP protein was verified by western blot analysis, while the purity of COMP was more than 98% as identified in SDS-PAGE followed by Coomassie Brilliant Blue G250 staining (Figure S1). [17, 19, 20]

2.4 Western Blotting

Cells (1×10^6 cells per sample) subjected to different treatments or aortic tissue dissected from 8-week old mice were lysed in RIPA buffer and prepared for extraction of whole-cell protein samples. Then, equal amounts of total protein (50-100 μg) per sample were resolved using 8% or 10% SDS-PAGE and transferred onto nitrocellulose membranes. The blots were subsequently incubated with primary antibodies (SM22α, α-SMA, calponin, GAPDH, COMP, cleaved Notch1 and Notch1; 1:1000) and IRDye-conjugated secondary antibodies (1:10000, Rockland Inc., Gilbertsville, PA, USA). The fluorescence signal was detected with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA), and the band density was analyzed using NIH Image J software.

2.5 Quantitative PCR Analysis

Total RNA was extracted from 5×10^5 cells per sample by using Trizol reagent (Life Technologies, Carlsbad, CA, USA), and equal amounts (2 μg) were reverse transcribed to cDNA by using 5× All-In-One RT MasterMix (TransGen Biotech, Beijing, China). Quantitative PCR amplification was performed using a QuantStudio 3 Real-Time PCR System (Stratagene Corp., La Jolla, CA, USA). SYBR Green 2× PCR mix (TransGen Biotech, Beijing, China) was used according to the manufacturer's instructions. The data were normalized to the internal control 18S. The primer sequences for the target genes are listed in Table S1.

2.6 Immunofluorescent Staining

Cells or tissue slides were first fixed by using 4% paraformaldehyde for 10 minutes, then incubated with rabbit anti-calponin or goat anti-SM22α antibody overnight at 4°C. Subsequently, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:400) (Life Technologies, Carlsbad, FL, USA) or Alexa Fluor 555-conjugated donkey anti-goat IgG (1:400) (Life Technologies, Carlsbad, FL, USA) antibody was applied, and the samples were incubated for 1 hour. Nuclei were stained with Hoechst 33342 for 1 minute. As a negative control, rabbit or goat IgG was applied instead of the primary antibodies. The fluorescence signal was visualized by confocal laser scanning microscopy (Leica Microsystems, Wetzlar, Germany).

2.7 Cell Contraction Assay

A Cell Contraction Assay kit (CBA-021) was purchased from Cell Biolabs Inc. (San Diego, CA, USA). The contractile ability of the cells was evaluated according to the manufacturer's instructions. Cells were harvested and resuspended in culture medium at density of 2×10^6 cells/ml. A collagen lattice was prepared by mixing cell suspension and ice-cold collagen gel solution in a volume ratio of 1:4. Then, 0.5 ml of the cell-collagen

mixture per well was added to a 24-well plate and incubated for 1 hour at 37°C. After collagen polymerization, 1.0 ml of culture medium was added on top of each collagen gel lattice. Twenty-four hours later, the 24-well plate was photographed, and the size of the collagen gel in each well was measured with NIH Image J software.

2.8 Animal Preparation

All animal studies and experimental procedures in the present study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Peking University Health Science Center and were performed in compliance with the ethical guidelines of that institution. Eight-week old C57BL/6J mice (Vital River, Beijing, China) were used for the *in vivo* ESC-VSMC differentiation assay. *COMP*$^{-/-}$ mice on a C57BL/6J background were kindly provided by Professor Äke Oldberg (Department of Cell and Molecular Biology, University of Lund, Sweden) [21]. *COMP*$^{-/-}$ mice and their wild-type (WT) littermates at 8 weeks old were euthanized by CO_2 for aorta isolation.

2.9 *In vivo* ESC-VSMC Differentiation

ESCs (1×10^6 cells in 50 μl of αMEM) were labeled with CM-DiI dye (Life Technologies, Carlsbad, FL, USA), mixed with 150 μl of Matrigel (BD Biosciences ,San Diego, CA, USA) containing PDGF-BB (10 ng/ml) or COMP (10 μg/ml) at 4°C, and then subcutaneously injected into C57BL/6 mice (male, 8 weeks old). After 13 days, the mice were euthanized, and the implants (Matrigel plugs) were embedded in OCT compound and frozen in liquid nitrogen. Sections (8 μm thick) were cut every 40 μm along the longitudinal axis of each Matrigel plug. In total, 8 interval sections were collected from each Matrigel plug for further immunofluorescent staining.

2.10 RNA Sequencing

Total RNA was isolated from undifferentiated ESCs or COMP-differentiated (3 days) ESCs (1×10^6 cells per sample) using GeneJET RNA Purification Kit (K0731, Thermo Scientific Inc., Rockford, CA, USA). After extensive quality control, 1 μg of RNA was used for generation of sequencing library using the TruSeq RNA Library Prep Kit V2 (RS-122-2002, Illumina) according to manufacturer's instructions. All libraries were sequenced on the NextSeq500 sequencer (FC-404-2005, Illumina) using the 35nt paired-end sequencing protocol. After sequencing, reads were aligned to genomes of mm9 by Subread (R version 1.24.2).

Uniq reads were kept and assigned to in-build refseq gene annotation of Rsubread using feature Counts. Differential analysis was conducted with Limma. Genes with fold change greater than 2 and FDR q value less than 0.05 were taken as significantly differentially expressed genes.

2.11 Lentivirus Packaging

Mouse COMP shRNA lentivirus were packaged as previously described [18]. The sequence of the mouse COMP shRNA was as follows: TGCTGTTGACAGTGAGCGCGAAGAATGACGATCAGAAAGATAGTGAAGCCACAGA TGTATCTTTCTGATCGTCATTCTTCTTGCCTACTGCCTCGGA.

Mouse Notch1/2/3 shRNA lentivirus were packaged using the "all-in-one" system. Briefly, a DNA oligo encoding Notch1/2/3 shRNA or negative control shRNA (SHC016) was designed via the RNAi consortium collection (MISSION® shRNA, www.sigmaaldrich.com). The DNA fragment was cloned into the pLKO vector. Then, the created lentivirus plasmids were co-transfected with virus package vectors including pLP1 and pLP2 into 293T cells by use of Lipofectamine 2000 (Invitrogen, Eugene, OR, USA). The medium was changed after 6 hours. Following 48 hours of culture, supernatant was collected as lentivirus stock for further infection of ESCs.

Dominant-negative MAML fragment (dnMAML, aa 13-74) fused with GFP (dnMAML-GFP) and COMP N terminus (aa 1-83) inserted with Flag-tag following the signal peptide (aa 1-19) (COMP sig-Flag-N terminus) overexpression lentivirus were packaged using a Gateway Technology kit following the manufacturer's instruction (Invitrogen, Eugene, OR, USA).

The Notch shRNA oligo sequences are presented in Table S2, while the primers for subcloning dnMAML-GFP and COMP sig-Flag-N-terminus are referred to Table S3.

2.12 Notch Pathway-responsive Luciferase Reporter Assays

The plasmids, including pcDNA3.1-Notch1, pcDNA3.1-Jagged1 and pGL3-4×CBF1-luc, were generously donated by Dr. Tao Wang from the University of Manchester, United Kingdom [22]. The JetPEI DNA transfection reagent (Polyplus-transfection, Strasbourg, France) was applied to transfect 293A cells with pcDNA3.1-Notch1, pGL3-4×CBF1-luc and pcDNA3.1-β-galactosidase together with pcDNA3.1-COMP, pcDNA3.1-Jagged1 or pcDNA3.1 vector. Forty-eight hours after transfection, transfected cells were harvested for measurement of luciferase activity using a Luciferase Assay System (Promega, Madison, MI, USA) according to the manufacturer's instructions. Meanwhile, the activity of β-galactosidase was evaluated via the ortho-Nitrophenyl-β-galactoside assay. Activation of the Notch pathway was represented as relative luminescence units normalized to β-galactosidase activity.

2.13 Co-immunoprecipitation

COMP-induced differentiated ESCs or plasmid-transfected COS-7 cells confluent in one 100 mm dish were lysed and then incubated with antibodies (1 μg per sample in 500 μl volume) against COMP, Notch1 or Flag-tag before being immunoprecipitated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The precipitated proteins were resolved using 10% SDS-PAGE and then incubated with antibodies against Notch1 or COMP. Rabbit or mouse IgG served as a negative control according to the species of primary antibodies.

2.14 Mammalian Two-hybrid Assay

Fragments encoding serial functional domains of human Notch1 EGF repeats [EGF1-10 (aa 20-410), EGF11-20 (aa 412-789), EGF21-30 (aa 791-1181), EGF31-36 (aa 1183-4278), EGF11-12 (aa 412-488), EGF13-14 (aa 489-563), EGF15-17 (aa 564-664) and EGF18-20 (aa 665-789)] were amplified by PCR and subcloned into pBIND vector. cDNA inserts encoding the mouse COMP N-terminal domain (aa 1-83) were subcloned into the pACT vector [18]. 293A cells were cotransfected with the target and bait constructs together with the luciferase

reporter plasmid pG5luc at a ratio of 1:1:1. Forty-eight hours after transfection, the cells were harvested, and cell lysates were used for luciferase activity assays with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The primers for subcloning the Notch1 EGF repeat fragments are provided in Table S3.

2.16 Statistical Analysis

All the results of the experimental studies were expressed as the mean ± SEM. Statistical analysis was performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA). For the statistical comparisons, we first evaluated whether the data were normally distributed. Then, the Brown-Forsythe test was used to check for similar variances among normally distributed data, after which we used Student's *t*-test for pairwise comparisons or ANOVA for comparisons of more than two groups if the test for similar variances was passed. In all cases, statistical significance was concluded if the two-tailed probability was less than 0.05. Details of statistical analysis for each experiment are presented in the corresponding figure legends.

3. Results

3.1 Purified COMP Directs ESC Differentiation into VSMCs *in vitro* and *in vivo*

To explore the role of COMP in ESC differentiation, we first purified COMP protein from 293A cells stably overexpressing mouse COMP and validated the purity of COMP protein in SDS-PAGE through Coomassie Brilliant Blue G250 staining (Supplemental Fig. 1). Then we seeded the mouse ESC line D3 on plates coated with 0.5 ml/cm^2 of Tris-buffered saline containing various concentrations of purified COMP protein (5 μg/ml, 10 μg/ml, 20 μg/ml) or collagen IV (10 μg/ml) and cultured the D3 cells in the absence of leukemia inhibitory factor for 7 days consecutively. Consisted with previous reports that collagen IV directly induced ESC differentiation into VSMCs [15], ESCs plated on collagen IV exhibited markedly elevated protein levels of VSMC markers (α-SMA, calponin and SM22α) (Fig. 1A). Similarly, COMP coating dose-dependently upregulated the protein expression of VSMC markers (α-SMA, calponin and SM22α) (Fig. 1A). Accordingly, COMP (10 μg/ml) time-dependently upregulated the VSMC contractile apparatus at both the protein and mRNA levels (Fig. 1B and Supplemental Fig. 2A). *Especially, COMP significantly upregulated smoothelin, the gene specifically expressed in VSMCs rather than myofibroblast [23, 24], excluding the directed differentiation towards myofibroblast.* By contrast, COMP did not direct ESC differentiation into endoderm (SOX17, FOXA2 and MIXL1), ectoderm (ZIC1 and SOX1), non-VSMC mesoderm lineages (cardiomyocytes: RYR2 and SERCA2a; endothelial cells: CD31, CD144 and CD34) or other mesenchymal lineages (adipocytes: DKL1; osteoblasts: RUNX2 and OSX1) (Supplemental Fig. 2B-F). Immunofluorescent staining further verified the mature differentiated VSMC phenotype, characterized by high expression of calponin and SM22α (Fig. 1C-D). Functionally, a collagen contractile assay revealed that differentiated cells displayed contractility, which was comparable with that of PDGF-BB-induced differentiated VSMCs, whereas no contraction was observed in non-differentiated ESCs (Fig. 1E). To further confirm the effect of COMP *in vivo*, we subcutaneously injected C57BL/6J mice with Matrigel including DiI-labelled ESCs and purified COMP protein (10 μg/ml) (Fig. 1F). Similar to PDGF-BB, COMP induced abundant expression of calponin and SM22α after 14 days in differentiated cells in Matrigel injected into mice, indicating the differentiation towards VSMCs *in vivo* (Fig. 1G and Supplemental Fig. 2G). Together, purified COMP is sufficient to drive ESC differentiation into VSMCs both *in vitro* and *in vivo*.

3.2 COMP is Necessary for ESC Differentiation into VSMCs

We next asked whether the endogenous COMP was involved in the differentiation into VSMCs. We applied three established differentiation systems, including collagen IV [5] (Fig. 2A), PDGF-BB [25] (Fig. 2B) and TGF-β [26] (Fig. 2C) induction for 7 days, to detect the protein expression of COMP at different time points. As a result, COMP was time-dependently upregulated in all three differentiation processes. Then, we silenced COMP by infection of COMP shRNA lentivirus into ESCs. Interestingly, COMP silencing significantly inhibited collagen IV- and PDGF-BB-induced VSMC differentiation as evidenced by reduced α-SMA, calponin and SM22α expression at both protein and mRNA levels (Fig. 2D-E and Supplemental Fig. 3). Thus, endogenous COMP also plays a critical role in the differentiation of ESCs into VSMCs.

3.3 Notch Signaling Mediates COMP-induced ESC Differentiation into VSMCs

To identify how COMP guided ESC differentiation towards VSMCs, we performed comparative RNA-Sequencing between ESCs and COMP-induced differentiated cells at day 3, the early stage of the differentiation process (Supplemental Fig. 4A). As the results, COMP upregulated 1,160 genes mainly related to pathways in cancer, Rap1 signaling pathway and Ras signaling pathway, but downregulated 898 genes including measles, osteoclast differentiation, toxoplasmosis, etc. (Fig. 3A and Supplemental Fig. 4B-C). The signaling activation of integrin, TGFβ, PDGF-BB, and Notch have been reported to mediate ESC-VSMC differentiation [7]. Interestingly, COMP greatly upregulated the expression of genes related to Notch signaling rather than the genes involved in the activation of other signals (Fig. 3B). Further real-time PCR analysis validated the upregulation of genes related to Notch signaling (Supplemental Fig. 4D). Canonical Notch signaling is initiated by ligand binding that leads to intramembrane proteolysis by a γ-secretase complex and releases the intracellular domain of Notch (NICD). Upon Notch activation, NICD forms a transcriptional complex with CSL/RBP-J and Mastermind-like (MAML) proteins and subsequently activates the transcription of Notch target genes, e.g., Hes1, Hey1 and Hey2 [27]. Indeed, COMP markedly enhanced the expression of these target genes at various time points during differentiation (Supplemental Fig. 5A). Conversely, COMP silencing during the process of collagen IV-induced differentiation repressed the induction of Notch target genes expression (Supplemental Fig. 5B). Overall, these results showed that COMP activates Notch signaling.

We next wondered whether Notch signaling mediated COMP-directed differentiation. Firstly, the γ-secretase inhibitor DAPT was applied to inhibit Notch signaling (Supplemental Fig. 6A). As shown in Fig. 3C-D and Supplemental Fig. 6B, DAPT application significantly inhibited COMP-evoked ESC-VSMC differentiation and cell contractility. Moreover, we created a dominant-negative MAML (dnMAML) lentivirus encoding a GFP-fused N-terminal NICD-binding domain of Mastermind-like1 (MAML1) that blocks MAML1-mediated recruitment of coactivators and downstream transcriptional activation of all Notch receptors [28]. Similarly, ESCs infected with the lentivirus encoding dnMAML exhibited inhibition of Notch signaling (Supplemental Fig. 6C) and COMP-induced ESC-VSMC differentiation (Fig. 3E and Supplemental Fig. 6D). Thus, COMP-activated Notch signaling mediates the differentiation of ESCs towards VSMCs. In accordance, COMP deficiency markedly attenuated VSMC contractile apparatus as well as cleaved Notch1 (indicating the activation of Notch1), Hes1, and Hey2 expression in the aortas compared with those of WT mice (Fig. 3F and Supplemental Fig. 7), indicating that COMP activated Notch signaling and induced VSMC differentiation *in vivo*.

3.4 COMP Directly Activates Notch1 signaling

The Notch family of cell surface receptors consists of four members, Notch1-4 [29, 30]. As previously reported, Notch1-3, but not Notch4, regulate VSMC development and differentiation [31-34]. To further verify which Notch member is the main contributor to the effect of COMP, we separately infected ESCs with lentivirus encoding Notch1, Notch2 or Notch3 shRNA (Supplemental Fig. 8). Only Notch1 silencing blocked COMP-induced upregulation of VSMC markers expression, whereas the silencing of Notch2 and Notch3 displayed no effect (Fig. 4A). To further identify the specific effect of COMP on Notch1, we measured cleaved Notch1 by western blot. Interestingly, COMP induced the elevation of

cleaved Notch1 as early as 6 to 24 hours of treatment in ESCs (Fig. 4B). Meanwhile, the expression of Notch1 and its 5 classic ligands displayed no change during 24-hour stimulation (Supplemental Fig. 9), indicating that COMP did not transcriptionally regulate Notch1 or its ligands to activate Notch1 signaling in the short term. Moreover, we applied a CBF1 response luciferase reporter system in 293A cells to further evaluate the effect of COMP on Notch1 activation. Similar to Jagged1, a canonical ligand of Notch1, COMP dose-dependently enhanced luciferase reporter transcription and activated Notch1 signaling (Fig. 4C). Moreover, the overexpression of COMP also increased the cleavage of Notch1 in 293A cells (Fig. 4D). Meanwhile, the mRNA levels of Notch1 and its ligands were not altered by COMP (Supplemental Fig. 10). These results suggest that COMP directly and rapidly activates Notch1 signaling independently of transcriptional regulation. To further verify the specificity of direct effect of COMP on Notch1 activation in the process of ESC-VSMC differentiation, we applied another differentiation induction reagent collagen IV in the CBF1 response luciferase reporter system and found that collagen IV did not directly activate Notch1 signaling, implying the specific effect of COMP on directly activating Notch1 (Fig. 4E).

3.5 COMP Directly Interacts with Notch1

To further explore how COMP activated Notch1, we first performed a co-immunoprecipitation assay on COMP-induced differentiated VSMCs and COS-7 cells overexpressing Notch1 and COMP respectively. In both types of cells, Notch1 was similarly immunoprecipitated with COMP by anti-COMP antibody but not by rabbit IgG. Inversely, COMP was also exclusively immunoprecipitated with Notch1 by anti-Notch1 antibody (Fig. 5A-B). Next, four domains of COMP including the N-terminal domain [N; aa 1-83], the epidermal growth factor repeat domain [EGF; aa 84-261], the type III repeat domain [type III; aa 266-520] and the C-terminal domain [C; aa 521-755] were subcloned into separate Flag-CMV plasmids (Fig. 5C). COS-7 cells overexpressing Notch1 and various Flag-tagged domains of COMP were subjected to co-immunoprecipitation using anti-Flag antibody. We found that Notch1 was specifically immunoprecipitated by the Flag-tagged N-terminal domain but not by other COMP fragments (Fig. 5D), indicating that COMP interacted with Notch1 through its N-terminus. In turn, we applied a mammalian two-hybrid assay to identify the COMP binding domain of Notch1. Since the EGF domain of Notch1 is the usual binding site for its extracellular ligand proteins [35], we constructed pBIND plasmids containing the following distinct fragments of the Notch1 EGF domain: aa 20 to 410 (EGF1-10), aa 412 to 789 (EGF11-20), aa 791 to 1181 (EGF21-30), and aa 1183 to 1426 (EGF31-36) (Fig. 5E). These pBIND plasmids were individually cotransfected into 293A cells along with a pACT vector encoding the COMP N-terminal domain. As shown in Figure 5F, cells transfected with the EGF11-20 domain exhibited the highest luciferase activity compared with other EGF domains of Notch1, indicating that EGF11-20 contains the potential binding domain of COMP. We further defined the binding motif of Notch1 by subcloning the EGF11-12, EGF13-14, EGF15-17 and EGF18-20 domains (Fig. 5E), and found that EGF11-12 mediated COMP interaction with Notch1 (Fig. 5G). Thus, COMP binds to the EGF11-12 domain of Notch1 through its N-terminal domain.

3.6 The N-terminal Domain of COMP is Sufficient to Activate Notch1 Signaling and

Guide ESC Differentiation towards VSMCs

We next asked whether the N-terminal domain of COMP alone could exhibit effects comparable to those of COMP in directing Notch1 activation and ESC-VSMC differentiation. We infected ESCs with control lentivirus or lentivirus encoding Flag-N-terminal domain, and further validated the N-terminus was overexpressed and extracellularly secreted by infected cells (Supplemental Fig. 11A-B). As expected, Flag-N-terminal domain overexpression significantly increased cleaved Notch1 and upregulated Notch1 target genes expression on day 7 during the differentiation process, indicating the activation of Notch1 signaling (Fig. 6A-B). Meanwhile, overexpression of Flag-N-terminal domain alone induced ESC-VSMC differentiation, which was comparable to the effect of COMP induction (Figure 6C-E and Supplemental Fig. 11C). Thus, the N-terminal fragment of COMP independently activates Notch1 signaling and induces the differentiation of ESCs into VSMCs.

4. Discussion

In the present study, we identified a novel role of the ECM protein COMP in dictating ESC differentiation into VSMCs. Mechanistically, COMP may serve as a novel Notch1 ligand to directly activate Notch1 signaling. COMP-driven ESC-VSMC differentiation may provide significant prospects for new regenerative therapies to promote the recovery of impaired VSMCs, as well as for understanding the pathogenesis of vascular diseases.

Our major finding is that COMP is attributable to the ECM microenvironment for ESC-VSMC differentiation during development dependent on Notch1 signaling. Especially, not only exogenous COMP protein, but endogenous COMP is also involved in this process. We found that endogenous COMP was upregulated during the differentiation of stem cells into SMCs, in accordance with previous microarray data [4], whereas silencing endogenous COMP inhibits ESC-VSMC differentiation induced by collagen IV or PDGF-BB, indicating that endogenous COMP is generally essential for ESC-VSMC differentiation. ESC differentiation towards the smooth muscle lineage not only regulates vascular development but also contributes to the postnatal pathogenesis of vascular diseases. Although *COMP*$^{-/-}$ mice are viable and exhibit no significant abnormality of vascular development, postnatal *COMP*$^{-/-}$ VSMCs exhibited a decreased VSMC contractile apparatus and reduced contractility in the absence of pathological stimuli, and *COMP*$^{-/-}$ mice are more susceptible than WT mice to atherosclerotic neointima formation and medial calcification [16-18, 36, 37]. Herein, we found that COMP deficiency attenuated the VSMC contractile apparatus in aortas as well. Taken together, COMP deficiency significantly retarded ESC-VSMC differentiation, it is conceivable that the absence of COMP at least partially impaired ESC differentiation towards the smooth muscle lineage during the prenatal stage.

Moreover, both silencing of endogenous COMP and COMP deficiency exhibited the attenuated Notch activation during ESC-VSMC differentiation *in vitro* and in postnatal aortas *in vivo*, respectively. In mammals, there are four Notch receptors (Notch1-4) and five canonical ligands (Jagged1 and 2, Delta-like 1, 3, and 4) [27]. Notch signaling plays an essential role in vascular development and postnatal arteriogenesis. Human Notch genes are linked to various vascular genetic diseases, such as Alagille syndrome [38], Adams-Oliver syndrome [39] and CADASIL [40]. In particular, Notch1-3 and Jagged1 have been identified as predominant during the development and differentiation of VSMCs [34, 41, 42]. Inhibition of Notch signaling by DAPT attenuates stem cell–VSMC differentiation *in vitro* [4]. Together with our finding that endogenous COMP silencing inhibits Notch activation and ESC-VSMC differentiation, we speculated that endogenous COMP-Notch pathway may play a critical role in ESC-VSMC differentiation. *Although Notch receptors may have some functional redundancy, they cannot replace one another at least due to their spatial and temporal expression. Notch2 and Notch3 are the predominant isoforms in mature VSMCs, which exhibit a comparable low level of Notch1 expression [40]. Especially, Notch2 is indispensable in VSMCs of large-caliber vessels, while Notch3 play a major role in VSMCs of smaller caliber vessels lacking of Notch2 expression [33, 43]. In current study, we explored that Notch1-related signals rather than Notch2/3-related signals mediated COMP-induced ESC-VSMC differentiation. Thus, Notch2/3 may contribute to the late/mature stage of ESC-VSMC differentiation when Notch1 turns into a low level of expression. To confirm this speculation needs further investigation.*

Besides activating Notch1 signaling, another interesting finding of current study is that COMP is a non-canonical ligand of the Notch1 receptor. COMP directly associates with and activates Notch1 to induce the differentiation of ESCs into SMCs, without altering the expression of other canonical ligands. By using co-immunoprecipitation and mammalian two-hybrid assays, we identified the EGF11-12 domain on the extracellular motif of Notch1 as the binding site of COMP; this domain is also responsible for the binding of canonical ligands (e.g., Jagged1 and DLL4) to Notch1 [44]. To further clarify whether COMP directly or indirectly activated Notch1 by affecting the interaction of Notch1 with other canonical ligands, we assessed Notch1 activation by using a luciferase assay and western blot analysis in the exogenous transfection cell system. Our data suggested that COMP did not significantly affect or even exhibit a trend toward attenuating Jagged1 activation of Notch1 (data not shown). Moreover, other differentiation inducer, such as collagen IV, was not able to directly activate Notch1, indicating the specific role of COMP in Notch1 activation (Figure 5F). Thus, COMP is a novel non-canonical Notch1 ligand. Mice with complete blockage of Notch signaling by knock-in of dnMAML in neural crest cells exhibited suppressed VSMC development and differentiation [32]. Loss of global Notch1 function results in early embryonic lethality due primarily to a vascular defect [45]. Herein, we discovered that *COMP*$^{-/-}$ mice exhibited the downregulation of VSMC contractile apparatus in aortas as well as the decreased activation of Notch1, implying that COMP might serve as a novel agonist of Notch1 in regulation of VSMC differentiation *in vivo*. Interestingly, *COMP*$^{-/-}$ mice do not display the abnormality in vascular development as existed in mice with Notch1 inactivation, indicating that Notch1 signaling does not only mediate COMP effects on VSMC differentiation, but also might be involved in other process in vascular development. Moreover, Notch1 canonical ligand Jagged1 also directed stem cell-VSMC differentiation [4]. Further comparison of COMP and canonical ligands in Notch1 activation may favor to explain the different phenotypes of *COMP*$^{-/-}$ mice and *Notch1*$^{-/-}$ mice.

Notably, several non-canonical Notch ligands have been identified in mammals, including integral membrane-bound and glycosylphosphatidylinositol (GPI)-linked proteins (e.g., Delta-like 1, contactin 1), the ECM protein CCN3, thrombospondin (TSP)-2 and TSP-4 [29, 46-48]. COMP belongs to the TSP matricellular protein family and is also known as TSP-5. Our previous studies and others have identified several proteins that bind to COMP and specifically mediate its effects in various physiological and pathological conditions [49]. In the cardiovascular system, COMP binds to thrombin via EGF repeats [50], BMP-2 via type III repeats [16] and integrin β1 and β3 via the C-terminal domain [18, 51] and subsequently fine-tunes hemostasis, vascular calcification, dilated cardiomyopathy and atherosclerosis, respectively. Herein, we further verified that the N-terminal domain of COMP could mimic the inducible effect of COMP in ESC differentiation into VSMCs, which is favorable to the development of new regenerative therapies to repair injured VSMCs in vascular diseases.

Taken together, COMP is a potential Notch1 receptor and drives ESC differentiation towards the smooth cell lineage through directly activating Notch1 signaling. Thus, COMP may be potentially and therapeutically applied in vascular regeneration to recover the injured VSMCs in vascular diseases.

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Conflict of Interest

None.

References

[1] Q. Xiao, Z. Luo, A.E. Pepe, A. Margariti, L. Zeng, Q. Xu, Embryonic stem cell differentiation into smooth muscle cells is mediated by Nox4-produced H2O2, Am J Physiol Cell Physiol 296(4) (2009) C711-23.

[2] Q. Xiao, A.E. Pepe, G. Wang, Z. Luo, L. Zhang, L. Zeng, Z. Zhang, Y. Hu, S. Ye, Q. Xu, Nrf3-Pla2g7 interaction plays an essential role in smooth muscle differentiation from stem cells, Arterioscler Thromb Vasc Biol 32(3) (2012) 730-44.

[3] E.M. Maguire, Q. Xiao, Q. Xu, Differentiation and Application of Induced Pluripotent Stem Cell-Derived Vascular Smooth Muscle Cells, Arterioscler Thromb Vasc Biol 37(11) (2017) 2026-2037.

[4] K. Kurpinski, H. Lam, J. Chu, A. Wang, A. Kim, E. Tsay, S. Agrawal, D.V. Schaffer, S. Li, Transforming growth factor-beta and notch signaling mediate stem cell differentiation into smooth muscle cells, Stem Cells 28(4) (2010) 734-42.

[5] Q. Xiao, L. Zeng, Z. Zhang, Y. Hu, Q. Xu, Stem cell-derived Sca-1+ progenitors differentiate into smooth muscle cells, which is mediated by collagen IV-integrin alpha1/beta1/alphav and PDGF receptor pathways, Am J Physiol Cell Physiol 292(1) (2007) C342-52.

[6] A.E. Pepe, Q. Xiao, A. Zampetaki, Z. Zhang, A. Kobayashi, Y. Hu, Q. Xu, Crucial role of nrf3 in smooth muscle cell differentiation from stem cells, Circ Res 106(5) (2010) 870-9.

[7] N.M. Kane, Q. Xiao, A.H. Baker, Z. Luo, Q. Xu, C. Emanueli, Pluripotent stem cell differentiation into vascular cells: a novel technology with promises for vascular re(generation), Pharmacol Ther 129(1) (2011) 29-49.

[8] H. Wang, X. Luo, J. Leighton, Extracellular Matrix and Integrins in Embryonic Stem Cell Differentiation, Biochem Insights 8(Suppl 2) (2015) 15-21.

[9] D. Soteriou, B. Iskender, A. Byron, J.D. Humphries, S. Borg-Bartolo, M.C. Haddock, M.A. Baxter, D. Knight, M.J. Humphries, S.J. Kimber, Comparative proteomic analysis of supportive and unsupportive extracellular matrix substrates for human embryonic stem cell maintenance, J Biol Chem 288(26) (2013) 18716-31.

[10] K. Narayanan, V.Y. Lim, J. Shen, Z.W. Tan, D. Rajendran, S.C. Luo, S. Gao, A.C. Wan, J.Y. Ying, Extracellular matrix-mediated differentiation of human embryonic stem cells: differentiation to insulin-secreting beta cells, Tissue Eng Part A 20(1-2) (2014) 424-33.

[11] F.M. Watt, W.T. Huck, Role of the extracellular matrix in regulating stem cell fate, Nat Rev Mol Cell Biol 14(8) (2013) 467-73.

[12] A. Didangelos, X. Yin, K. Mandal, M. Baumert, M. Jahangiri, M. Mayr, Proteomics characterization of extracellular space components in the human aorta, Mol Cell Proteomics 9(9) (2010) 2048-62.

[13] G. Suna, W. Wojakowski, M. Lynch, J. Barallobre-Barreiro, X. Yin, U. Mayr, F. Baig, R. Lu, M. Fava, R. Hayward, C. Molenaar, S.J. White, T. Roleder, K.P. Milewski, P. Gasior, P.P. Buszman, P. Buszman, M. Jahangiri, C.M. Shanahan, J. Hill, M. Mayr, Extracellular Matrix Proteomics Reveals Interplay of Aggrecan and Aggrecanases in Vascular Remodeling of Stented Coronary Arteries, Circulation 137(2) (2018) 166-183.

[14] R.M. Simpson, X. Hong, M.M. Wong, E. Karamariti, S.I. Bhaloo, D. Warren, W. Kong, Y. Hu, Q. Xu, Hyaluronan Is Crucial for Stem Cell Differentiation into Smooth Muscle Lineage, Stem Cells 34(5) (2016) 1225-38.

[15] M. Sone, H. Itoh, J. Yamashita, T. Yurugi-Kobayashi, Y. Suzuki, Y. Kondo, A. Nonoguchi, N. Sawada, K. Yamahara, K. Miyashita, K. Park, M. Shibuya, S. Nito, S. Nishikawa, K. Nakao, Different differentiation kinetics of vascular progenitor cells in primate and mouse embryonic stem cells, Circulation 107(16) (2003) 2085-8.

[16] Y. Du, Y. Wang, L. Wang, B. Liu, Q. Tian, C.J. Liu, T. Zhang, Q. Xu, Y. Zhu, O. Ake, Y. Qi, C. Tang, W. Kong, X. Wang, Cartilage oligomeric matrix protein inhibits vascular smooth muscle calcification by interacting with bone morphogenetic protein-2, Circ Res 108(8) (2011) 917-28.

[17] L. Wang, J. Zheng, Y. Du, Y. Huang, J. Li, B. Liu, C.J. Liu, Y. Zhu, Y. Gao, Q. Xu, W. Kong, X. Wang, Cartilage oligomeric matrix protein maintains the contractile phenotype of vascular smooth muscle cells by interacting with alpha(7)beta(1) integrin, Circ Res 106(3) (2010) 514-25.

[18] Y. Fu, C. Gao, Y. Liang, M. Wang, Y. Huang, W. Ma, T. Li, Y. Jia, F. Yu, W. Zhu, Q. Cui, Y. Li, Q. Xu, X. Wang, W. Kong, Shift of Macrophage Phenotype Due to Cartilage Oligomeric Matrix Protein Deficiency Drives Atherosclerotic Calcification, Circ Res 119(2) (2016) 261-76.

[19] L. Wang, J. Zheng, X. Bai, B. Liu, C.J. Liu, Q. Xu, Y. Zhu, N. Wang, W. Kong, X. Wang, ADAMTS-7 mediates vascular smooth muscle cell migration and neointima formation in balloon-injured rat arteries, Circ Res 104(5) (2009) 688-98.

[20] C.J. Liu, W. Kong, K. Ilalov, S. Yu, K. Xu, L. Prazak, M. Fajardo, B. Sehgal, P.E. Di Cesare, ADAMTS-7: a metalloproteinase that directly binds to and degrades cartilage oligomeric matrix protein, Faseb j 20(7) (2006) 988-90.

[21] L. Svensson, A. Aszodi, D. Heinegard, E.B. Hunziker, F.P. Reinholt, R. Fassler, A. Oldberg, Cartilage oligomeric matrix protein-deficient mice have normal skeletal development, Mol Cell Biol 22(12) (2002) 4366-71.

[22] T. Wang, C.M. Holt, C. Xu, C. Ridley, P.O.J. R, M. Baron, D. Trump, Notch3 activation modulates cell growth behaviour and cross-talk to Wnt/TCF signalling pathway, Cell Signal 19(12) (2007) 2458-67.

[23] F.T. van der Loop, G. Schaart, E.D. Timmer, F.C. Ramaekers, G.J. van Eys, Smoothelin,

a novel cytoskeletal protein specific for smooth muscle cells, J Cell Biol 134(2) (1996) 401-11.

[24] F.T. van der Loop, G. Gabbiani, G. Kohnen, F.C. Ramaekers, G.J. van Eys, Differentiation of smooth muscle cells in human blood vessels as defined by smoothelin, a novel marker for the contractile phenotype, Arterioscler Thromb Vasc Biol 17(4) (1997) 665-71.

[25] M. Wanjare, F. Kuo, S. Gerecht, Derivation and maturation of synthetic and contractile vascular smooth muscle cells from human pluripotent stem cells, Cardiovasc Res 97(2) (2013) 321-30.

[26] A. Wang, Z. Tang, X. Li, Y. Jiang, D.A. Tsou, S. Li, Derivation of smooth muscle cells with neural crest origin from human induced pluripotent stem cells, Cells Tissues Organs 195(1-2) (2012) 5-14.

[27] R. Kopan, M.X. Ilagan, The canonical Notch signaling pathway: unfolding the activation mechanism, Cell 137(2) (2009) 216-33.

[28] I. Maillard, U. Koch, A. Dumortier, O. Shestova, L. Xu, H. Sai, S.E. Pross, J.C. Aster, A. Bhandoola, F. Radtke, W.S. Pear, Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells, Cell Stem Cell 2(4) (2008) 356-66.

[29] B. D'Souza, L. Meloty-Kapella, G. Weinmaster, Canonical and non-canonical Notch ligands, Curr Top Dev Biol 92 (2010) 73-129.

[30] S. Artavanis-Tsakonas, M.D. Rand, R.J. Lake, Notch signaling: cell fate control and signal integration in development, Science 284(5415) (1999) 770-6.

[31] H. Uyttendaele, V. Closson, G. Wu, F. Roux, G. Weinmaster, J. Kitajewski, Notch4 and Jagged-1 induce microvessel differentiation of rat brain endothelial cells, Microvasc Res 60(2) (2000) 91-103.

[32] L. Chang, M. Noseda, M. Higginson, M. Ly, A. Patenaude, M. Fuller, A.H. Kyle, A.I. Minchinton, M.C. Puri, D.J. Dumont, A. Karsan, Differentiation of vascular smooth muscle cells from local precursors during embryonic and adult arteriogenesis requires Notch signaling, Proc Natl Acad Sci U S A 109(18) (2012) 6993-8.

[33] Q. Wang, N. Zhao, S. Kennard, B. Lilly, Notch2 and Notch3 function together to regulate vascular smooth muscle development, PLoS One 7(5) (2012) e37365.

[34] P.O. Guichet, S. Guelfi, M. Teigell, L. Hoppe, N. Bakalara, L. Bauchet, H. Duffau, K. Lamszus, B. Rothhut, J.P. Hugnot, Notch1 stimulation induces a vascularization switch with pericyte-like cell differentiation of glioblastoma stem cells, Stem Cells 33(1) (2015) 21-34.

[35] K. Hori, A. Sen, S. Artavanis-Tsakonas, Notch signaling at a glance, J Cell Sci 126(Pt 10) (2013) 2135-40.

[36] M. Wang, Y. Fu, C. Gao, Y. Jia, Y. Huang, L. Liu, X. Wang, W. Wang, W. Kong, Cartilage oligomeric matrix protein prevents vascular aging and vascular smooth muscle cells senescence, Biochem Biophys Res Commun 478(2) (2016) 1006-13.

[37] Y. Du, C. Gao, Z. Liu, L. Wang, B. Liu, F. He, T. Zhang, Y. Wang, X. Wang, M. Xu, G.Z. Luo, Y. Zhu, Q. Xu, X. Wang, W. Kong, Upregulation of a disintegrin and metalloproteinase with thrombospondin motifs-7 by miR-29 repression mediates vascular smooth muscle calcification, Arterioscler Thromb Vasc Biol 32(11) (2012) 2580-8.

[38] C. Fouillade, M. Monet-Lepretre, C. Baron-Menguy, A. Joutel, Notch signalling in smooth muscle cells during development and disease, Cardiovasc Res 95(2) (2012) 138-46.

[39] A.B. Stittrich, A. Lehman, D.L. Bodian, J. Ashworth, Z. Zong, H. Li, P. Lam, A. Khromykh, R.K. Iyer, J.G. Vockley, R. Baveja, E.S. Silva, J. Dixon, E.L. Leon, B.D. Solomon, G. Glusman, J.E. Niederhuber, J.C. Roach, M.S. Patel, Mutations in NOTCH1 cause Adams-Oliver syndrome, Am J Hum Genet 95(3) (2014) 275-84.

[40] J.T. Baeten, B. Lilly, Notch Signaling in Vascular Smooth Muscle Cells, Adv Pharmacol 78 (2017) 351-382.

[41] A. Bhattacharyya, S. Lin, M. Sandig, K. Mequanint, Regulation of vascular smooth muscle cell phenotype in three-dimensional coculture system by Jagged1-selective Notch3 signaling, Tissue Eng Part A 20(7-8) (2014) 1175-87.

[42] J.T. Baeten, A.R. Jackson, K.M. McHugh, B. Lilly, Loss of Notch2 and Notch3 in vascular smooth muscle causes patent ductus arteriosus, Genesis 53(12) (2015) 738-48.

[43] Y. Wang, L. Pan, C.B. Moens, B. Appel, Notch3 establishes brain vascular integrity by regulating pericyte number, Development 141(2) (2014) 307-17.

[44] I. Rebay, R.J. Fleming, R.G. Fehon, L. Cherbas, P. Cherbas, S. Artavanis-Tsakonas, Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor, Cell 67(4) (1991) 687-99.

[45] F.P. Limbourg, K. Takeshita, F. Radtke, R.T. Bronson, M.T. Chin, J.K. Liao, Essential role of endothelial Notch1 in angiogenesis, Circulation 111(14) (2005) 1826-32.

[46] K. Sakamoto, S. Yamaguchi, R. Ando, A. Miyawaki, Y. Kabasawa, M. Takagi, C.L. Li, B. Perbal, K. Katsube, The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway, J Biol Chem 277(33) (2002) 29399-405.

[47] H. Meng, X. Zhang, K.D. Hankenson, M.M. Wang, Thrombospondin 2 potentiates notch3/jagged1 signaling, J Biol Chem 284(12) (2009) 7866-74.

[48] E.J. Benner, D. Luciano, R. Jo, K. Abdi, P. Paez-Gonzalez, H. Sheng, D.S. Warner, C. Liu, C. Eroglu, C.T. Kuo, Protective astrogenesis from the SVZ niche after injury is

controlled by Notch modulator Thbs4, Nature 497(7449) (2013) 369-73.

[49] Y. Fu, W. Kong, Cartilage Oligomeric Matrix Protein: Matricellular and Matricrine Signaling in Cardiovascular Homeostasis and Disease, Curr Vasc Pharmacol 15(3) (2017) 186-196.

[50] Y. Liang, Y. Fu, R. Qi, M. Wang, N. Yang, L. He, F. Yu, J. Zhang, C.H. Yun, X. Wang, J. Liu, W. Kong, Cartilage oligomeric matrix protein is a natural inhibitor of thrombin, Blood 126(7) (2015) 905-14.

[51] Y. Huang, J. Xia, J. Zheng, B. Geng, P. Liu, F. Yu, B. Liu, H. Zhang, M. Xu, P. Ye, Y. Zhu, Q. Xu, X. Wang, W. Kong, Deficiency of cartilage oligomeric matrix protein causes dilated cardiomyopathy, Basic Res Cardiol 108(5) (2013) 374.

Figures and Figure Legends

Fig. 1. COMP directly induces ESC differentiation into VSMCs. Cell culture dishes were pre-coated with 0.5 ml of Tris-buffered saline containing various concentrations of mouse collagen IV or mouse COMP per cm^2 area at room temperature overnight. Undifferentiated ESCs (2×10^5 cells per 60 mm dish) were seeded on mouse-collagen IV (10 μg/ml)- or purified-mouse-COMP-coated dishes in differentiation medium. (A) Representative western blot analysis and quantification of α-SMA, calponin and SM22α expression in undifferentiated ESCs and ESCs cultured for 7 days on dishes coated with different doses of COMP or collagen IV (Col IV). GAPDH was used as the internal control. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. control. One-way ANOVA followed by the Dunnett's multiple comparisons test. (B) Representative western blot analysis and quantification of α-SMA, calponin and SM22α expression in undifferentiated ESCs and ESCs seeded on COMP (10 μg/ml)-coated plates for various periods. GAPDH was measured as the internal control. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. 0 day. One-way ANOVA followed by the Dunnett's multiple comparisons test. (C-D) Immunofluorescent staining of calponin (C) and SM22α (D) in undifferentiated ESCs and ESCs seeded on COMP (10 μg/ml)-coated plates for 7 days. Scale bar, 20 μm. (E) ESCs with or without 7-day treatment of COMP (10 μg/ml) or PDGF-BB (10 ng/ml) were harvested and mixed with ice-cold collagen gel solution in a volume ratio of 1:4. Twenty-four hours after collagen polymerization, the size of the collagen gel in each well was evaluated. **P*<0.05 vs. control. One-way ANOVA followed by the Dunnett's multiple comparisons test. n=4. (F) Schematic diagram of subcutaneous injection of Matrigel solution including DiI-labelled ESCs together with differentiation induction reagents into 8-week old C57BL/6J mice. (G) Following 14-day induction by COMP (10 μg/ml) or PDGF-BB (10 ng/ml) in Matrigel plugs in mice, cross-sections of isolated plugs were analyzed through immunofluorescent staining of calponin. Scale bar, 10 μm.

Fig. 2. Endogenous COMP is involved in the process of differentiation of ESCs into VSMCs. Cell culture dishes were pre-coated with 0.5 ml of Tris-buffered saline containing 10 μg/ml of mouse collagen IV or 1% of gelatin per cm^2 area at room temperature overnight. Undifferentiated ESCs (2×10^5 cells per 60 mm dish) were seeded on mouse-collagen IV-coated dishes in differentiation medium for differentiation into VSMCs. For PDGF-BB and TGF-β induction, undifferentiated ESCs were seeded on 1% gelatin-coated dishes in differentiation medium followed by PDGF-BB (10 ng/ml) or TGF-β (10 ng/ml) treatment. (A-C) Representative western blot analysis and quantification of COMP expression during ESC differentiation induced by collagen IV (A, 10 μg/ml), PDGF-BB (B, 10 ng/ml) or TGF-β (C, 10 ng/ml). GAPDH was used as the internal control. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. 0 day. One-way ANOVA followed by the Dunnett's multiple comparisons test. (D-E) ESCs were infected with lentivirus encoding control or COMP shRNA. Following differentiation induced by 7-day treatment of collagen IV (D, 10 μg/ml) or PDGF-BB (E, 10 ng/ml), western blotting was performed to evaluate COMP, α-SMA, calponin and SM22α expression. GAPDH was used as the loading control. Quantification of protein expression was from three independent experiments. Data are presented as the mean ± SEM. **P*<0.05 vs. control shRNA, two-tailed paired Student's *t*-test.

Fig. 3. Notch signaling mediates COMP-induced ESC differentiation into VSMCs. (A) Volcano plots of genes in RNA-Sequencing between ESCs and COMP (10 μg/ml)-differentiated cells (3 days). Blue plots indicate the downregulated genes, and red plots indicate the upregulated genes. (B) Heatmap comparative analysis of genes related to Notch, TGFβ, PDGF-BB and integrin signals between ESCs and COMP (10 μg/ml)-differentiated cells (3 days). (C) Representative western blot analysis and quantification of α-SMA, calponin and SM22α in ESCs seeded on the plates coated with COMP (10 μg/ml) in the absence or presence of DAPT (10 μM) for 7 days. GAPDH was applied as the loading control. Vehicle represents DMSO applied as solvent control. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. vehicle. Two-tailed paired Student's *t*-test. (D) ESCs were induced to differentiate with or without treatment of COMP (10 μg/ml) in the absence or presence of DAPT (10μM) for 7 days. Vehicle represents DMSO applied as a solvent control. Differentiated cells were harvested and mixed with ice-cold collagen gel solution in a volume ratio of 1:4. Twenty-four hours after collagen polymerization, the size of the collagen gel in each well was evaluated. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05, one-way ANOVA followed by the Dunnett's multiple comparisons test. (E) Representative western blot analysis and quantification of α-SMA, calponin and SM22α in ESCs infected with GFP or dnMAML lentivirus seeded on the plates coated with COMP (10 μg/ml) for 7 days. GFP-encoded lentivirus was applied as control. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. control. Two-tailed paired Student's *t*-test. (F) Representative western blot analysis and quantification of cleaved Notch1, α-SMA, calponin and SM22α in aortas from 8-week old WT and *COMP*$^{-/-}$ mice. Data are presented as the mean ± SEM. **P*<0.05 vs. control. Two-tailed unpaired Student's *t*-test. n=6.

Fig. 4. COMP directly activates Notch1 signaling. (A) Representative western blot analysis and quantification of α-SMA, calponin and SM22α expression in ESCs infected with lentivirus encoding control shRNA, Notch1 shRNA, Notch2 shRNA or Notch3 shRNA and then induced by COMP to differentiate for 7 days. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. control shRNA, one-way ANOVA followed by the Dunnett's multiple comparisons test. (B) Representative western blot analysis and quantification of cleaved Notch1 expression in ESCs seeded on COMP-coated plates at different time points. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. 0 hour. One-way ANOVA followed by the Dunnett's multiple comparisons test. (C) Luciferase activity assay of 293A cells transfected with 4×CBF1-Luc together with pcDNA3.1-COMP, pcDNA3.1-Notch1 or pcDNA3.1-Jagged1. β-Galactosidase was co-transfected as the internal control. **P*<0.05, data are presented as the mean ± SEM. One-way ANOVA followed by the Dunnett's multiple comparisons test. Three independent experiments were performed in triplicate. (D) Representative western blot and quantification of cleaved Notch1 in 293A cells transfected with pcDNA3.1-COMP, pcDNA3.1-Notch1 or pcDNA3.1-Jagged1. **P*<0.05, data are presented as the mean ± SEM. One-way ANOVA followed by the Dunnett's multiple comparisons test. Three independent experiments were performed in triplicate. (E) Twenty-four-well plates were pre-coated with 0.5 ml of Tris-buffered saline containing various concentrations of mouse collagen IV or mouse COMP per cm^2 area at room temperature overnight. 293A cells transfected with 4×CBF1-Luc and pcDNA3.1-Notch1 were seeded on the precoated plates for 48 hours. Luciferase activity was measured to indicate Notch1 activation. β-Galactosidase was co-transfected as the internal control. 293A cells seeded on the plates without coating served as control. **P*<0.05, data are presented as the mean ± SEM. One-way ANOVA followed by the Dunnett's multiple comparisons test. n=4.

Fig. 5. COMP directly interacts with Notch1. (A-B) Coimmunoprecipitation (Co-IP) of COMP and Notch1 in ESC-differentiated cells (A) and COS-7 cells overexpressing COMP and Notch1 (B). Rabbit IgG was used as a negative control for IP. (C) Schematic illustration of COMP constructs used to map the corresponding domains (N-terminus, EGF, Type III, and C-terminus). (D) Representative western blot of Notch1 immunoprecipitated from the lysates of COS-7 cells overexpressing Notch1 and Flag-fused COMP construct domains by anti-Flag antibody. Mouse IgG was served as a negative control for IP. (E) Schematic illustration of Notch1 extracellular EGF constructs. (F-G) Mammalian two-hybrid analysis of interaction between the COMP N-terminal domain and the Notch1 EGF domain. Luciferase activity assay of 293A cells transiently transfected with various domains of Notch1 EGF repeats subcloned into the pBIND vector, together with the N-terminal domain of COMP subcloned into the pACT vector. Data are presented as the mean ± SEM. $*P<0.05$ vs. vector. One-way ANOVA followed by the Dunnett's multiple comparisons test. Three independent experiments were performed in triplicate.

Fig. 6. The COMP N-terminal domain activates Notch1 signaling and guides ESC differentiation towards VSMCs. (A) Representative western blot analysis and quantification of cleaved Notch1 in ESCs infected with GFP or COMP N-terminal domain lentivirus. GFP-encoded lentivirus was applied as a control. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs control. Two-tailed paired Student's *t*-test. (B) Real-time PCR of Hes1, Hey1 and Hey2 in ESCs infected with control or COMP N-terminal domain lentivirus. Data are presented as the mean ± SEM. **P*<0.05 vs control. Two-tailed paired Student's *t*-test. Three independent experiments were performed in duplicate. (C) Representative western blot analysis and quantification of α-SMA, calponin and SM22α expression in ESCs infected with lentivirus encoding COMP N-terminal domain or seeded on COMP-coated plates for 7 days. Data are presented as the mean ± SEM from three independent experiments. **P*<0.05 vs. control. One-way ANOVA followed by the Dunnett's multiple comparisons test. (D-E) Immunofluorescent staining of calponin (D) and SM22α (E) in undifferentiated ESCs and ESCs overexpressing the N-terminal domain of COMP for 7 days. Scale bar, 20 μm.

Highlights:

- COMP guides ESC-VSMC differentiation dependent on Notch1 activation.
- COMP directly activates Notch1 signaling.
- COMP N-terminus directly interacts with the EGF11-12 domain of Notch1.

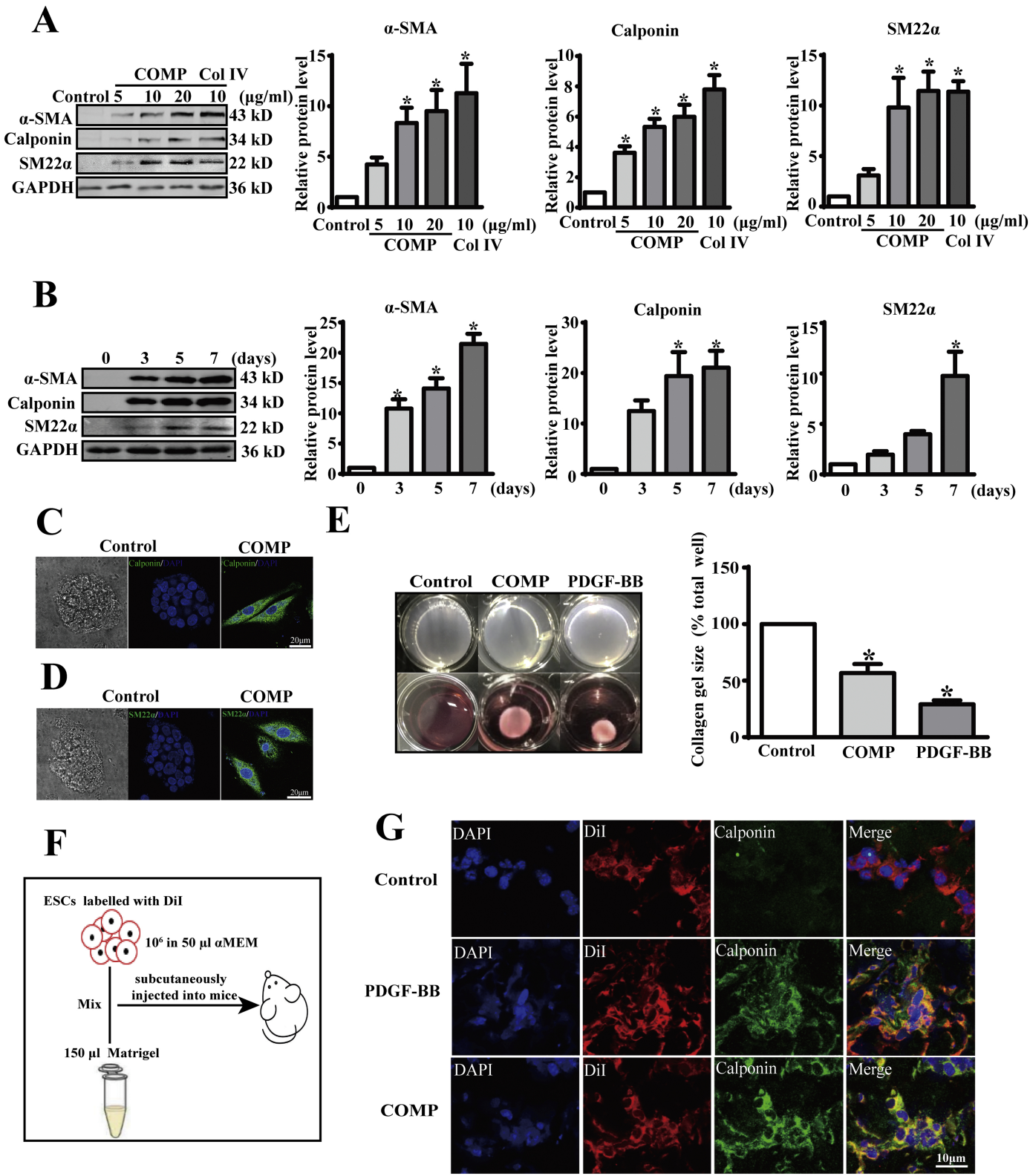
A
COMP Col IV
Control 5 10 20 10 (μg/ml)
α-SMA 43 kD
Calponin 34 kD
SM22α 22 kD
GAPDH 36 kD
α-SMA
Calponin
SM22α
Relative protein level
Control 5 10 20 10 (μg/ml)
COMP Col IV
B
0 3 5 7 (days)
α-SMA 43 kD
Calponin 34 kD
SM22α 22 kD
GAPDH 36 kD
α-SMA
Calponin
SM22α
Relative protein level
0 3 5 7 (days)
C
Control
COMP
Calponin/DAPI
20μm
D
Control
COMP
SM22α/DAPI
20μm
E
Control COMP PDGF-BB
Collagen gel size (% total well)
Control
COMP
PDGF-BB
F
ESCs labelled with DiI
10⁶ in 50 μl αMEM
Mix
subcutaneously injected into mice
150 μl Matrigel
G
Control
PDGF-BB
COMP
DAPI
DiI
Calponin
Merge
10μm

Figure 1

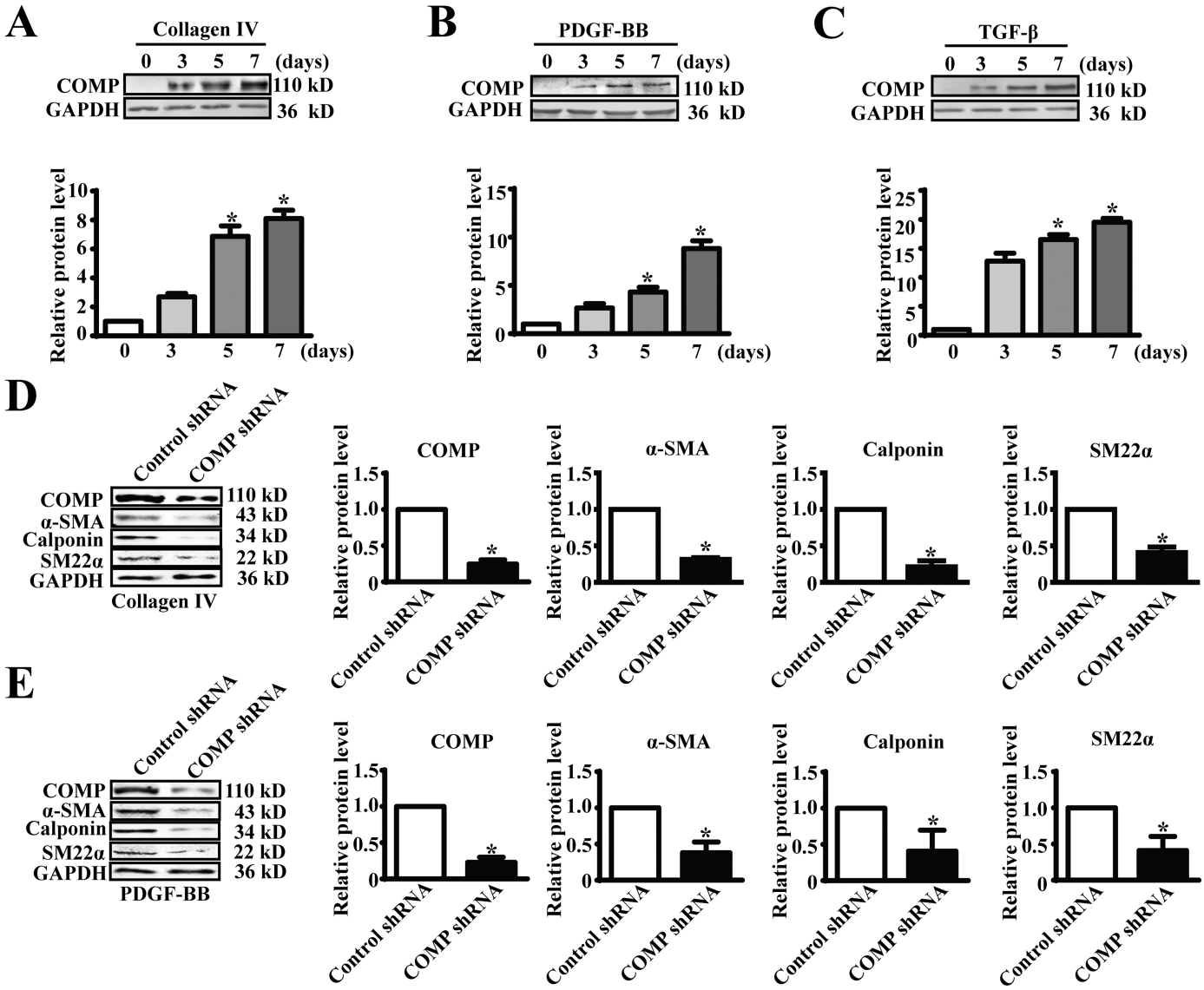
A
Collagen IV
0 3 5 7 (days)
COMP
GAPDH
110 kD
36 kD
Relative protein level
0 3 5 7 (days)
B
PDGF-BB
0 3 5 7 (days)
COMP
GAPDH
110 kD
36 kD
Relative protein level
0 3 5 7 (days)
C
TGF-β
0 3 5 7 (days)
COMP
GAPDH
110 kD
36 kD
Relative protein level
0 3 5 7 (days)
D
Control shRNA
COMP shRNA
COMP
α-SMA
Calponin
SM22α
GAPDH
110 kD
43 kD
34 kD
22 kD
36 kD
Collagen IV
COMP
α-SMA
Calponin
SM22α
Relative protein level
Control shRNA
COMP shRNA
E
Control shRNA
COMP shRNA
COMP
α-SMA
Calponin
SM22α
GAPDH
110 kD
43 kD
34 kD
22 kD
36 kD
PDGF-BB
COMP
α-SMA
Calponin
SM22α
Relative protein level
Control shRNA
COMP shRNA

Figure 2

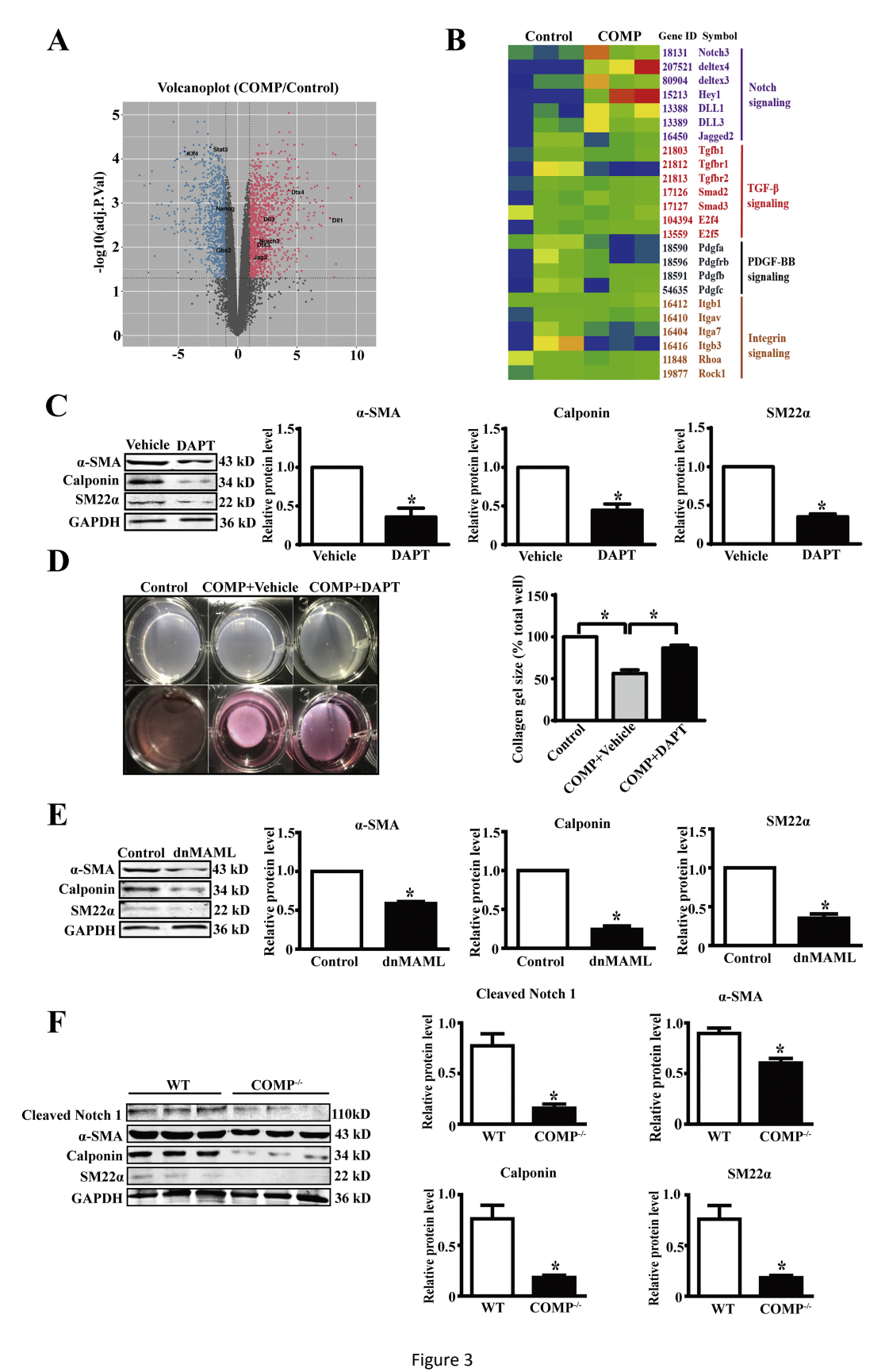
A
Volcanoplot (COMP/Control)
-log10(adj.P.Val)
Klf4
Stat3
Nanog
Dtx4
Dll3
Dll1
Notch3
Dtx1
Gbx2
Jag2
B
Control
COMP
Gene ID
Symbol
18131 Notch3
207521 deltex4
80904 deltex3
15213 Hey1
13388 DLL1
13389 DLL3
16450 Jagged2
Notch signaling
21803 Tgfb1
21812 Tgfbr1
21813 Tgfbr2
17126 Smad2
17127 Smad3
104394 E2f4
13559 E2f5
TGF-β signaling
18590 Pdgfa
18596 Pdgfrb
18591 Pdgfb
54635 Pdgfc
PDGF-BB signaling
16412 Itgb1
16410 Itgav
16404 Itga7
16416 Itgb3
11848 Rhoa
19877 Rock1
Integrin signaling
C
Vehicle
DAPT
α-SMA
Calponin
SM22α
GAPDH
43 kD
34 kD
22 kD
36 kD
Relative protein level
Vehicle
DAPT
D
Control
COMP+Vehicle
COMP+DAPT
Collagen gel size (% total well)
E
Control
dnMAML
F
WT
COMP-/-
Cleaved Notch 1
110kD

Figure 3

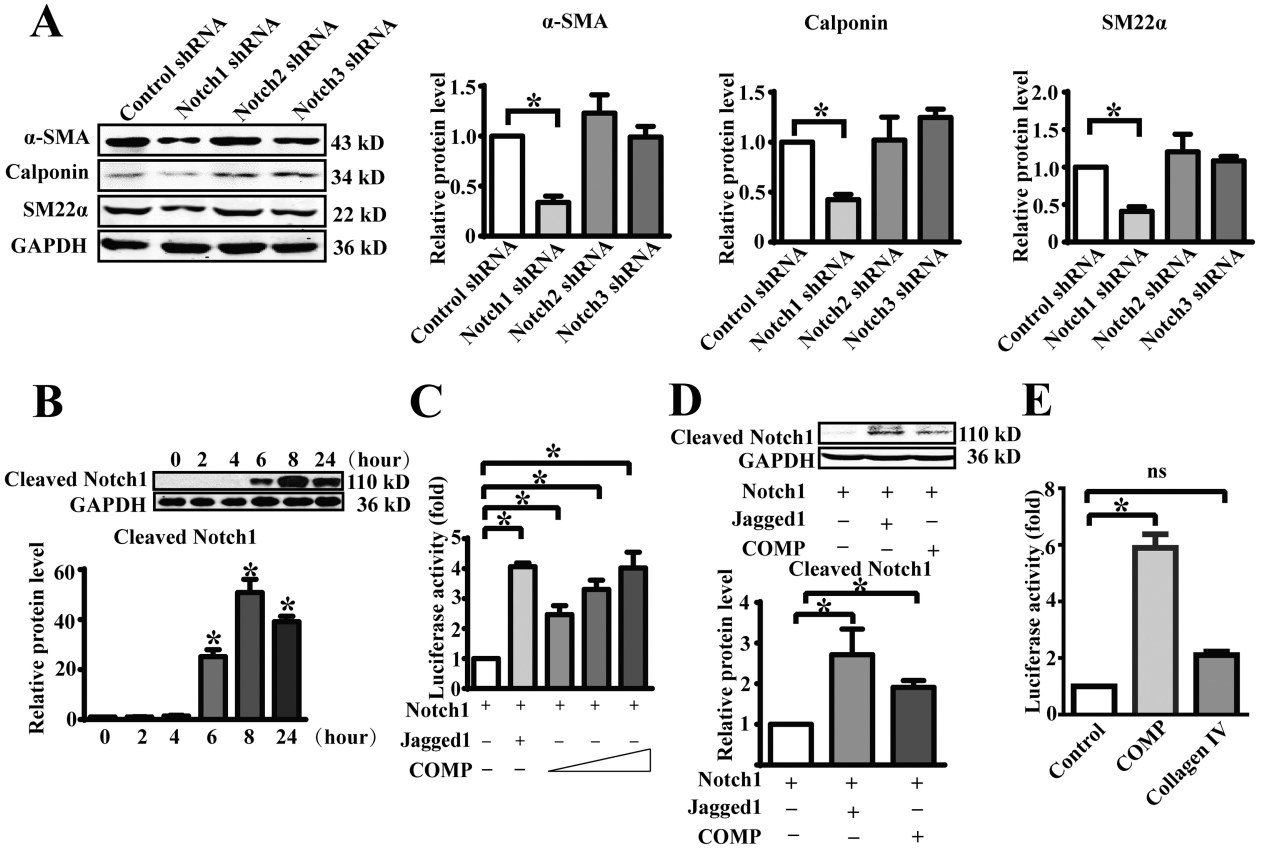
A
Control shRNA
Notch1 shRNA
Notch2 shRNA
Notch3 shRNA
α-SMA
Calponin
SM22α
GAPDH
43 kD
34 kD
22 kD
36 kD
α-SMA
Calponin
SM22α
Relative protein level
B
0 2 4 6 8 24 (hour)
Cleaved Notch1
110 kD
GAPDH
36 kD
Cleaved Notch1
Relative protein level
C
Luciferase activity (fold)
Notch1
Jagged1
COMP
D
Cleaved Notch1
110 kD
GAPDH
36 kD
Notch1
Jagged1
COMP
Cleaved Notch1
Relative protein level
E
ns
Luciferase activity (fold)
Control
COMP
Collagen IV

Figure 4

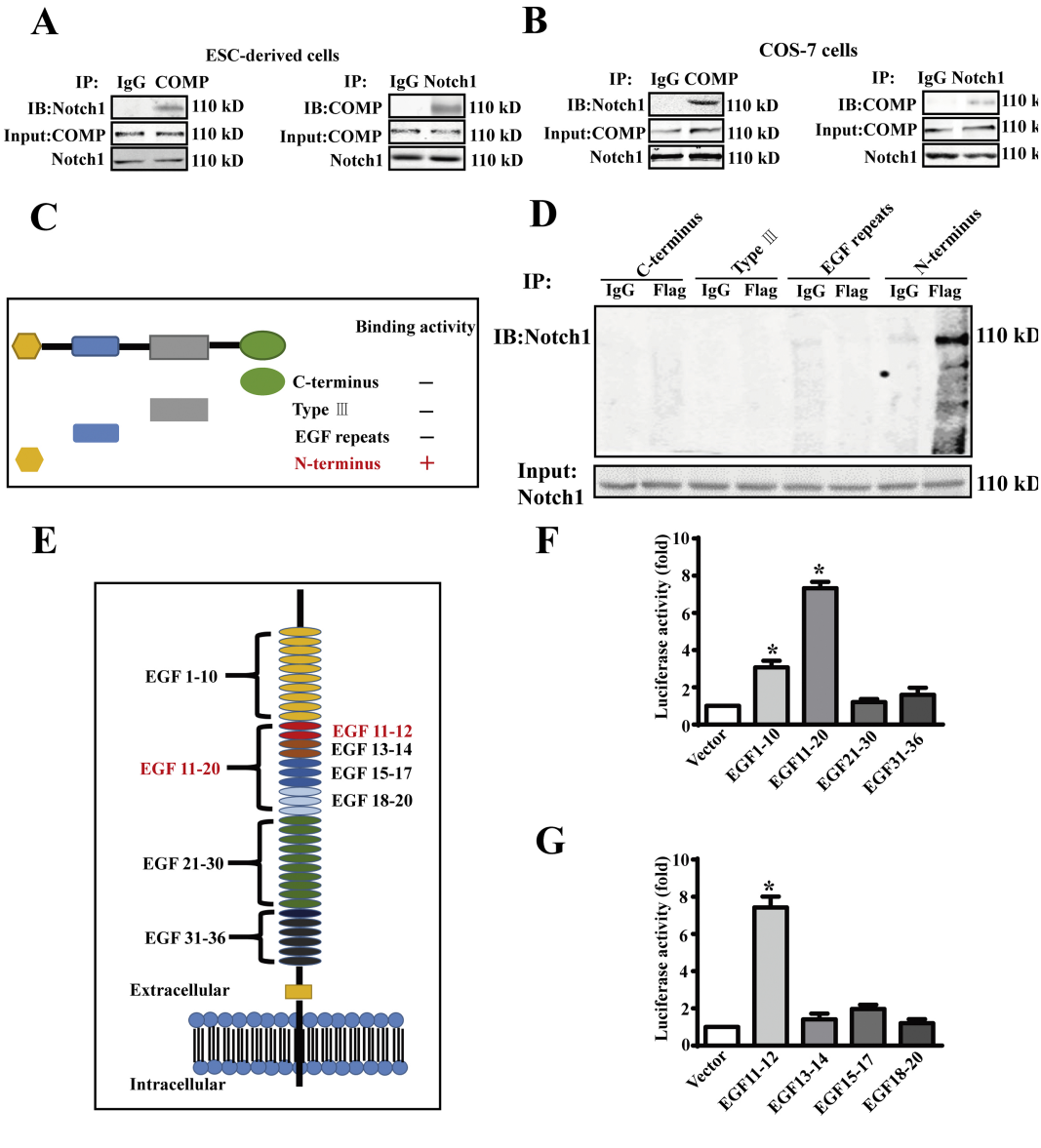
A
ESC-derived cells
IP: IgG COMP
IB:Notch1
Input:COMP
Notch1
110 kD
IP: IgG Notch1
IB:COMP
Input:COMP
Notch1
110 kD
B
COS-7 cells
IP: IgG COMP
IB:Notch1
Input:COMP
Notch1
110 kD
IP: IgG Notch1
IB:COMP
Input:COMP
Notch1
C
Binding activity
C-terminus –
Type Ⅲ –
EGF repeats –
N-terminus +
D
IP:
C-terminus
Type Ⅲ
EGF repeats
N-terminus
IgG Flag IgG Flag IgG Flag IgG Flag
IB:Notch1
110 kD
Input:
Notch1
110 kD
E
EGF 1-10
EGF 11-20
EGF 11-12
EGF 13-14
EGF 15-17
EGF 18-20
EGF 21-30
EGF 31-36
Extracellular
Intracellular
F
Luciferase activity (fold)
0
2
4
6
8
10
Vector
EGF1-10
EGF11-20
EGF21-30
EGF31-36
G
Luciferase activity (fold)
0
2
4
6
8
10
Vector
EGF11-12
EGF13-14
EGF15-17
EGF18-20

Figure 5

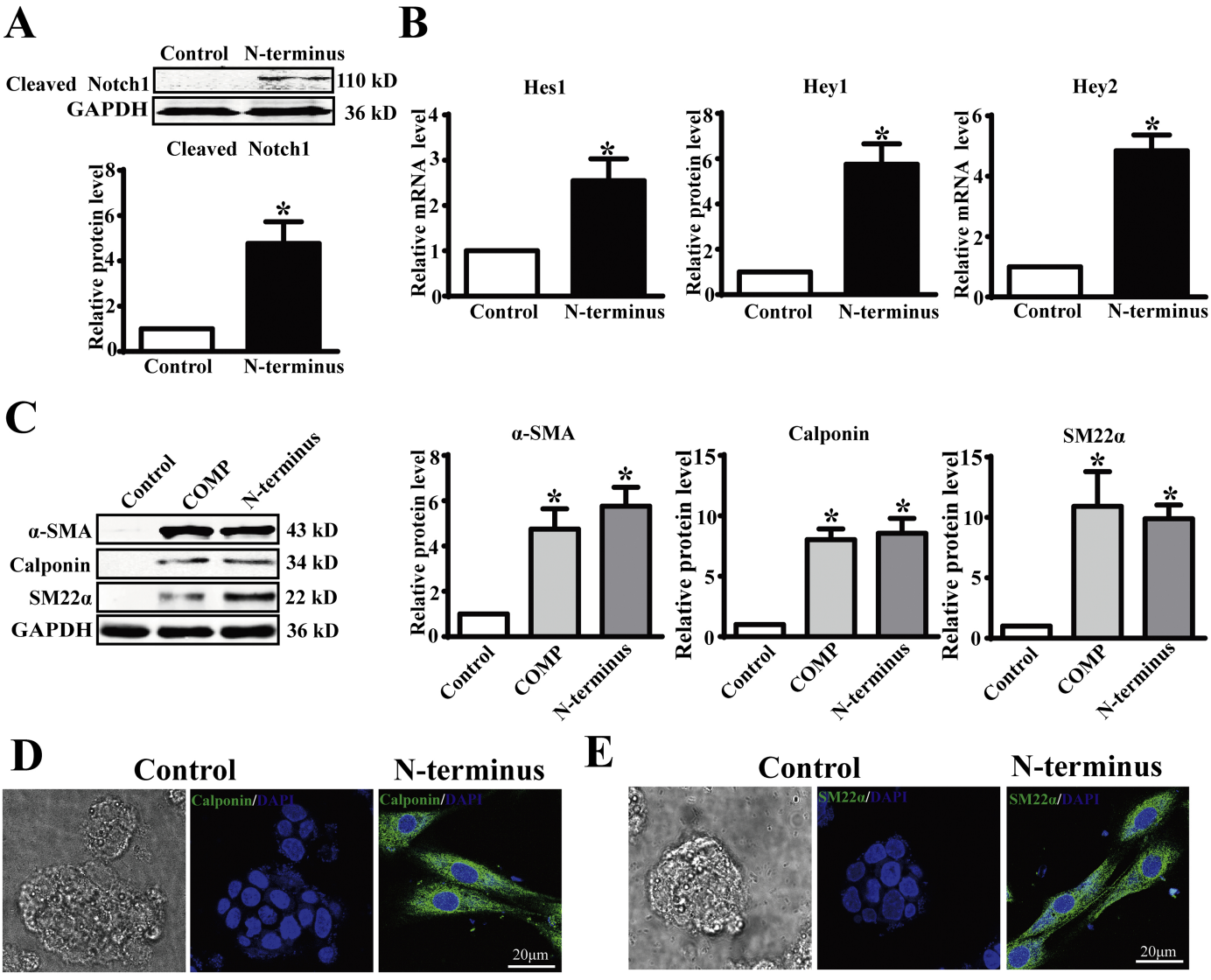
A
Control
N-terminus
Cleaved Notch1
110 kD
GAPDH
36 kD
Cleaved Notch1
Relative protein level
Control
N-terminus
B
Hes1
Relative mRNA level
Hey1
Relative protein level
Hey2
Relative mRNA level
C
Control
COMP
N-terminus
α-SMA
43 kD
Calponin
34 kD
SM22α
22 kD
GAPDH
36 kD
α-SMA
Calponin
SM22α
Relative protein level
D
Control
N-terminus
Calponin/DAPI
20μm
E
SM22α/DAPI
20μm

Figure 6